Universidade de Aveiro Departamento de Biologia

Ano 2016

Fátima da Conceição Ferreira dos Santos

Hazard assessment for Nickel nanoparticles in the soil: full life cycle test with *Enchytraeus crypticus*

Avaliação do perigo de nanoparticulas de Níquel no solo: ciclo de vida completo utilizando *Enchytraeus crypticus*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Mónica Amorim, investigadora auxiliar do CESAM, Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Susana Gomes, Investigadora em Pós-doutoramento do CESAM, Departamento de Biologia da Universidade de Aveiro.

Aos meus pais, ao meu irmão, aos amigos, Obrigada ♥

o júri

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Agradecimentos

Aos meus pais e irmão por todo o apoio, luta, e por me ensinarem e ajudarem a nunca desistir e a lutar pelos meus sonhos. Amanhã será sempre um dia melhor, se não for a gente faz acontecer!

Á Doutora Mónica Amorim um muito obrigada pela oportunidade e orientação aos longos destes anos.

A Susana Gomes e a Rita Bicho sem as quais, chegar a esta fase teria sido muito mais difícil. A vossa ajuda, paciência, compreensão, transmissão de conhecimentos, disponibilidade, cumplicidade….. palavras não chegam para descrever o sentimento de gratidão que tenho por vocês.

Ao Micael Gonçalves, meu parceiro de laboratório e amigo de muitas horas de trabalho, assim como a todos os restantes membros do grupo de investigação sempre presentes e disponíveis.

A todos os amigos que sempre estiveram presentes e aos amigos que ganhei neste fantástico percurso durante todos estes anos de aprendizagem e crescimento pessoal e profissional. Obrigada por todos os momentos e memórias que certamente não esquecerei e que sempre terão um espacinho grande no meu coração.

Palavras-chave Organismos de solo, Teste de ciclo de vida completo, *Enchytraeus crypticus*, Nanopartículas

Resumo

A nanotecnologia é uma área em constante crescimento, com cada vez mais produtos contendo nanopartículas (NPs) a serem introduzidos no mercado e no nosso dia-a-dia, desde cosméticos a baterias e dispositivos médicos. As características das NPs (composição química, tamanho, forma, reatividade) prometem novas aplicações e tornam estes materiais altamente desejáveis. Devido ao aumento de produção, as NPs irão eventualmente chegar ao meio ambiente de forma direta ou indireta, onde podem causar efeitos negativos nos diferentes compartimentos dos ecossistemas (solo, ar, água).

Apesar de haver estudos ecotoxicológicos que se focam em nanomateriais, diferentes resultados (e por vezes contraditórios) podem ser encontrados mesmo para NPs com a mesma composição química, o que se pode dever a influência de fatores bióticos e abióticos que podem alterar a biodisponibilidade e portanto a toxicidade desses materiais.

NPs metálicas, como o níquel (Ni) são atualmente utilizadas em diversas aplicações, no entanto os efeitos das nanopartículas de níquel (NiNPs) em organismos de solo ainda são pouco conhecidos.

No presente estudo, a toxicidade de NiNPs (em comparação com $NiNO_3$) para o organismo modelo de solo *Enchytraeus crypticus* (Oligochaeta) foi investigada com base no teste padrão Teste de Reprodução em Enquitreídeos (em inglês Enchytraeid Reprodução test - ERT) e no recentemente desenvolvido teste de ciclo de vida completo (FLC), que adiciona ao teste padrão, parâmetros como a eclosão, o crescimento e o tempo para atingir a maturidade.

No geral, $NiNO_3$ foi mais tóxico para o *E. crypticus* do que as NiNPs e a toxicidade parece ocorrer através de mecanismos diferenciados. Na exposição a $NiNO_3$, os efeitos foram visíveis na redução do número de juvenis eclodidos ao nível da eclosão e mantiveram-se ao longo de todos os parâmetros avaliados no ciclo de vida (crescimento, estado de maturação, sobrevivência e reprodução). Relativamente à exposição a NiNPs, a eclosão foi o parâmetro mais sensível (CE_{10} = 47 mg NiNPs/kg), mas os organismos sobreviveram e reproduziram-se a concentrações até 1800 mg NiNPs/kg, mostrando que o efeito observado na eclosão foi um atraso. Exposição a 100 mg Ni/kg causou efeitos similares a concentrações mais altas (1000 e 1800 mg NiNPs/kg) indicando um maior efeito associado ao tamanho nano. Os atuais resultados realçam a potencial falta de uma dose-resposta monótona (com base na massa) para a avaliação de perigo de NPs e consequentemente a exigência da revisão dos procedimentos para a avaliação de risco.

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Keywords Soil organism, Full life cycle test, *Enchytraeus crypticus*, Nanoparticles

Abstract

Nanotechnology is an area of increasing growth, with more and more products containing nanoparticles (NPs) being introduced in the market and used in our daily life, from cosmetics to batteries and medical devices. NPs characteristics (chemical composition, size, shape, reactivity) promises new applications and make these materials highly desirable. Due to the increase of production NPs will eventually reach the environment either directly or indirectly, where they may cause negative effects in different compartments of ecosystems (soil, air, water).

Although there are ecotoxicological studies that focus on nanomaterials, different (and sometimes contradictory) results can be found even with NPs of the same chemical composition, which may be due to the influence of abiotic and biotic factors that can affect the bioavailability and hence the toxicity of those materials

Metallic NPs, such as nickel (Ni) are currently used in many applications, however the effects of nickel nanoparticles (NiNPs) in soil organisms are still poorly known.

In the present work, the aim was to evaluate the toxicity of NiNPs (compared to $NiNO_3$) for soil model organism *Enchytraeus crypticus* (Oligochaeta) using the standard Enchytraeid reproduction test (ERT) and the newly developed full life cycle test (FLC), which adds to the standard the parameters hatching, growth and time to reach maturity.

$NiNO_3$ was more toxic to *E. crypticus* than NiNPs and toxicity seems to occur via differentiated mechanisms. For $NiNO_3$ exposure effects where visible in the reduced number of hatched juveniles and then remained throughout all the endpoints evaluated in the life cycle (growth, maturity status, survival and reproduction). Regarding exposure to NiNPs, hatching was the most sensitive endpoint (EC_{10} = 47 mg NiNPs/kg), but organisms survived and reproduced at concentrations up to 1800 mg NiNPs/kg, revealing that the effect in the hatching was a delay. Exposure to 100 mg NiNPs/kg caused similar effects to the higher concentrations (1000 and 1800 mg NiNPs/kg), indicating higher nano-particulate effect. These results highlights the potential lack of monotone dose-response (based on mass) for hazard assessment of NPs and hence the requirement of a revised procedure for Risk Assessment.

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Chapter 1

General introduction

1- General Introduction

1.1. Ecotoxicology: definition and (current) challenges

The term ecotoxicology was used for the first time in 1969 by René Truhaut (Twardowska, 2004) as the "the study of adverse effects of chemicals with the aim of protecting natural species and populations" and appeared with the same base principles as toxicology, i.e. experimental testing, analysis of dose-effect relationships, and estimation of effect concentrations, such as the exposure concentration at which 50% effect is observed within a certain period (EC_{50}), which was of great benefit for environmental regulation (Straalen, 2003). Ecotoxicology is a multidisciplinary science which integrates several fields: chemistry, biology, pharmacology, medicine, pharmacology, pathology, immunology, endocrinology, genomics, and proteomics (Twardowska, 2004).

Traditional ecotoxicology approaches focused on individual endpoints (e.g. survival, reproduction, growth), however link those endpoints with effects at population levels is rather difficult and subject to uncertainty (Connon et al., 2012). Thus, "modern" ecotoxicology must ideally adopt integrative approaches of integrate mechanistic responses at the different levels of biological organization (molecular, cellular, whole organism, population, community and ecosystem) anchored with chemical analysis (Connon et al., 2012). Once this is costly and time consuming, the investigation of key links between chemical/stressor exposure and responses at different levels of biological organization (biomarkers) can be a first step towards making ecotoxicology more effective in its goal.

Most of the existing standardized tests with soil invertebrates aim to study the effects on parameters such as survival and/or reproduction of the model organism selected, e.g. enchytraeids (ISO, 2005; OECD, 2004a), earthworms (ISO, 1998, 1993; OECD, 2004b, 1984), collembolans (OECD, 2009). However, in natural ecosystems, organisms might be exposed to stressors during their entire life cycle (Preston and Snell, 2001) and also, the different developmental stages of an organism can have different sensitivities to stressors

(Crane et al., 2010a; Hammers-Wirtz and Ratte, 2000) which can put into question the ecological relevance of the data generated with the current ecotoxicological tests (Preston and Snell, 2001).

In the past few years, studies evaluating other endpoints rather than the standard are arising in the literature, for instance, evaluating embryonic development (Druart et al., 2010; Iglesias et al., 2002, 2000); hatching (Gonçalves et al., 2015; Shoaib et al., 2009) and growth (Chen et al., 2011; Meyer et al., 2010; Wu et al., 2013; Zhu et al., 2010) for aquatic and terrestrial organisms. Once they cover life stages (e.g. embryos) often more sensitive than the adult stage, they are usually shorter in duration and thus are also cost-effective.

However, studies covering the full life cycle (FLC) of the organisms are still scarce and mostly performed in aquatic organism: in the copepod *Bryocamptus zschokkei* (Brown et al., 2003), in the rotifer *Brachionus Calyciflorus* (Preston and Snell, 2001), in several fish species: *Pimephales promelas*, *Oryzias latipes*, *O. javanicus, Gobiocypris rarus* and *Danio rerio* (Crane et al., 2010b; Imai et al., 2007; Segner et al., 2003; Seki et al., 2005; Zhong et al., 2005); the exception is the recently developed FLC test for the soil invertebrate *Enchytraeus crypticus* (Enchytraeidae: Oligochaeta) (Bicho et al., 2015).

Full life-cycle tests must incorporate aspects from embryonic, development and reproductive parameters , thus organisms must be exposed, at least, from hatching until reach maturity (Brown et al., 2003). In that way, the most sensitive life stages (those to which ecological relevance can be linked) should be covered (Preston and Snell, 2001) which can potentially provide some clarification about the mechanism of response to the stressor.

1.2. Test organism

Appropriate model organisms for soil quality are essential for a risk assessment evaluation, therefore they should fulfill several criteria, such as: they should play a key role in the soil ecosystem , having a relevant response for conclusions on the system level; have a widespread distribution in different ecosystems, enabling comparisons between systems;

occur in a large number, being widely available and their response should be recordable; they should be collectable and cultivable both in field and laboratory conditions; should be exposed to a variety of stress factors, through soil solution, solid phase, and the gaseous phase in soil; also they should be sufficiently sensitive to a wide range of environmental stresses but not so sensitive that they easily become extinct (Didden and Römbke, 2001). Enchytraeids fulfill these criteria and are used in laboratory ecotoxicology test for more than 40 years (Römbke and Moser, 2002).

There are about 950 species of enchytraeids described worldwide (Jänsch et al., 2005). Enchytraeids are saprophagous of the mesofauna of the litter layer and the upper mineral soil, with a limited digging ability compared to most earthworms, but they may improve the small-scale water and air management of soil, especially when populations are high (Jänsch et al., 2005). They are widespread in many substrates, like soils, organic remains or sediments (Filimonova and Pokarzhevskii, 2000) therefore they have a broad tolerance towards a variety of soil properties like pH, texture and organic matter content (Jänsch et al., 2005).

Enchytraeus crypticus (Westheide and Graefe, 1992) (Fig.1), is one of the species contemplated in the standard Enchytraeid Reproduction Test (ISO, 2005; OECD, 2004a). As model species in soil ecotoxicology, *E. crypticus* present several advantages in comparison to the larger species *E. albidus* (as stated in Castro-Ferreira et al. (2012)): has a higher reproductive rate, shorter generation time which allow a shorter test period, and broader tolerance range to different soil properties (pH, texture, and organic matter content). In addition a full life cycle test was recently developed for *E. crypticus* (Bicho et al., 2015).

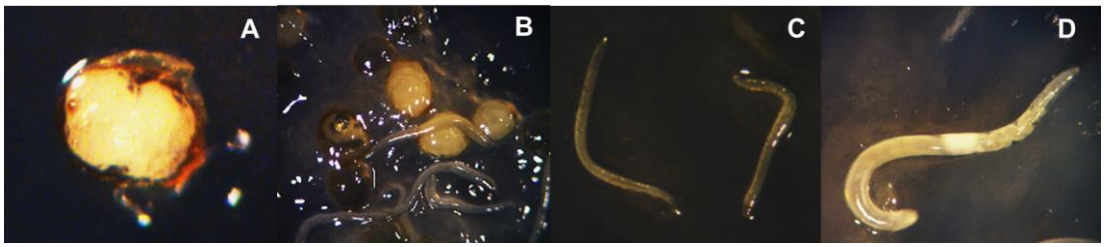

Fig 1. *Enchytraeus crypticus* in is life cycle: cocoon (A), hatched organisms and cocoons (B), juveniles (C) and mature adult with a well-developed clitellum (D).

1.3. Test chemicals: nickel nitrate ($NiNO_3$) and nickel-nanoparticles (NiNPs)

Nickel (Ni) is a metal of widespread distribution in the environment, being released through both natural (forest fires and vegetation, volcanic emissions and wind-blown dust) and anthropogenic sources, (burning of fossil fuels, spreading of sewage sludge and manure, and mining activities) (Liber et al., 2011; Phipps et al., 2002). It has several industrial applications like coinage manufacture, electroplating, nickel alloy production, spark plug and other ignition devices, as well as, electrical resistance heaters and batteries (Y. A. Iyaka, 2011). Ni is present in soils in concentrations from 3 up to 1000 mg Ni/kg soil (A. Y. Iyaka, 2011) and can reach 26000 mg/kg in heavily polluted areas (Alloway, 1995), being a compound of general concern in terms of environmental and human health protection (Broerse and van Gestel, 2010). Ni is an (semi-)essential element for several animal species, plants, and microorganisms. It contributes to lipid metabolism, hematopoiesis, and other biological functions at low concentrations, but has toxic effects on organisms above certain concentrations (Phipps et al., 2002).

Engineered (or non-natural) nanoparticles (NPs) are one of the most promising material science developments in recent times. Metallic NPs are among the most widely used type of nanomaterials (NMs) (Gallo et al., 2016; Vance et al., 2015). Nickel nanoparticles (NiNPs) characteristics include a high level of surface energy, high level of magnetism, large surface area, and low melting and burning points (Zhang et al., 2003). Therefore, NiNPs are being widely used in additives in ceramics, lubricants, and sintering; alloys; batteries; capacitor materials; catalysis reactions; ceramic and diamond tool production; electrical conductors / conductive paste; fuel cell applications; fuel combustion; magnetic materials; metallic conductive coatings; pigmentations and uranium purification (NiPERA, 2013).

NiNPs can be produced by combustion synthesis; mechanochemical processing; chemical precipitation; sol-gel processing; chemical vapor deposition; laser ablation and attrition and pyrolysis which are the most commonly used techniques (Stone et al., 2009). By 2013 the U.S. production of NiNPs was approximately 20 tons a year (NiPERA, 2013), thus NiNPs can eventually reach the environment.

1.4. Ecotoxicology of nanomaterials

1.4.1. Nanotechnology: definitions and applications

According to Buzea et al. (2007) "Nanotechnology can be defined as the design, synthesis, and application of materials and devices whose size and shape have been engineered at the nanoscale", thus it includes the development and production of nanosized engineered particles, fibres, coatings, and others, collectively referred to as nanomaterials (NMs) (Bleeker et al., 2013). Nanomaterial is, according to a recommendation by the European Commission (EU, 2011): "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm." "In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1% and 50% "(Fig 2.).

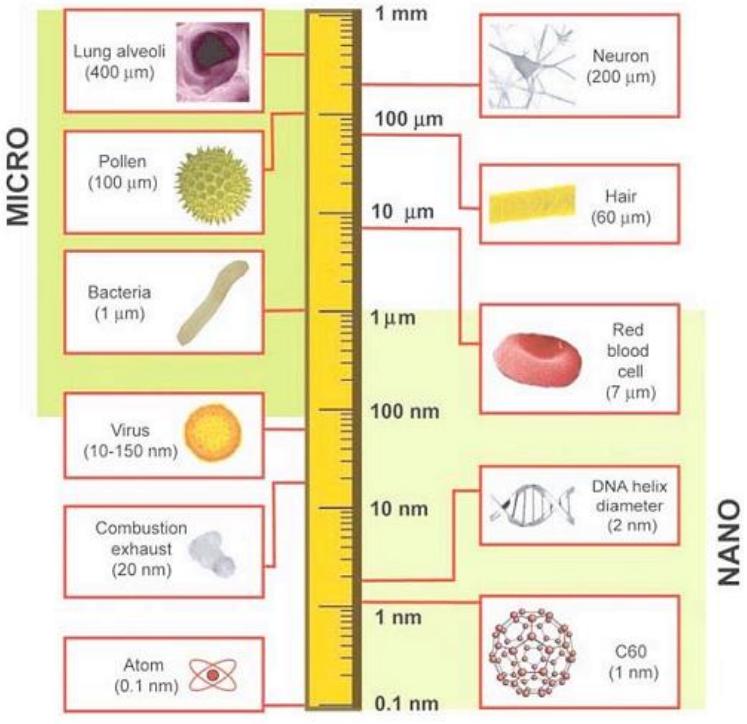

Fig 2. Length scale showing the nanometer in context. From Buzea et al., (2007)

Engineered NMs can be classified by their dimensionality and include: nano films or surface coating for the materials with one dimension at the nanoscale, two dimension nanostructures which include nanowires and nanotubes, and finally three dimension materials or NPs which include colloids, quantum dots and monocrystalline materials (Buzea et al., 2007). Besides size, other aspects to consider for the classification of NPs are morphology (e.g. spheres, wires, tubes, pyramids), composition (e.g. single material versus composites) and agglomeration state (Buzea et al., 2007).

Nanotechnology exploit the unique chemical, physical, electrical, and mechanical properties that emerge when matter is structured at the nanoscale, the subsequent resulting in new nano-products that have a vast range of applications including in medicine, electronics, energy production, cosmetics, sunscreens, coatings, batteries, fuel additives, paints, pigments, tires and cement among others (Lindquist et al., 2010; Piccinno et al., 2012; Vance et al., 2015; Yah et al., 2012).

Legislation regarding the production and use of NMs in the European Union are within the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) and the Classification, Labeling, and Packaging (CLP) regulations (Vance et al., 2015). Nevertheless metrics for nanomaterial characterization and labeling, and global standardized methods for risk evaluation are still need to be developed and refined (Vance et al., 2015).

1.4.2. Novel properties and entry in the environment

NPs can have different chemical, physical, and biological characteristics, and thus can behave differently, with respect to materials of a coarser structure, even when the elemental or molecular composition is the same (Bondarenko et al., 2013; Lövestam et al., 2010). Concerns regarding their increased production volumes and widespread use are being raised, as this can increase occupational and environmental exposure to the NPs/NMs (Bleeker et al., 2013; Bondarenko et al., 2013). The different routes of exposure to NMs/NPs can be dermal, by ingestion or inhalation (Fig 3.) (Vance et al., 2015).

Despite the growing concern, there are still major knowledge gaps for even the most used NPs, like the post-production life cycles (entry into the environment, environmental pathways, eventual environmental fate, and potential ecotoxicological effects) (Garner and Keller, 2014). Keller and Lazareva (2013) estimated that 17% of the produced NMs may be release to soils, which can happen during their use, by spillages, by intentional release, as an environmental remediation or as end-of-life waste (Garner and Keller, 2014).

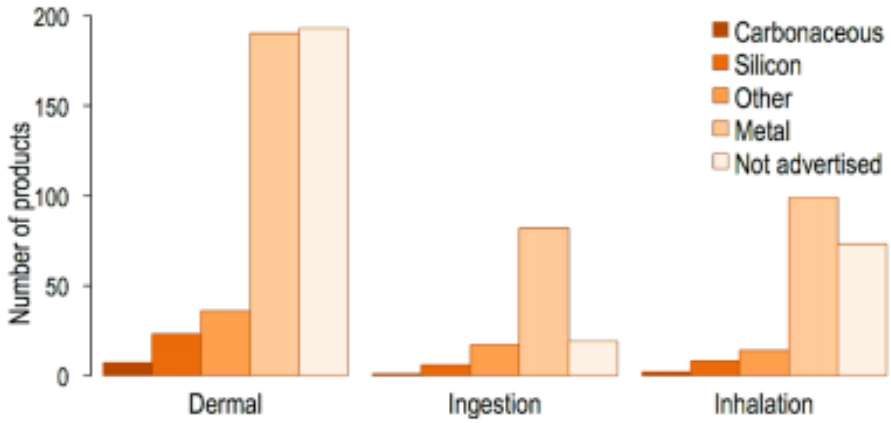

Fig 3. Potential exposure pathways of products containing nanomaterials. From Vance et al., (2015)

Some of the NPs properties can be extrapolated from the macro-scale, but others can change below a certain size. For instance, NMs have a much larger specific surface or interface area than coarser materials and for spherical NPs, the specific surface area increases with the inverse of the diameter (Lövestam et al., 2010). Other example is the size of the NPs, because small size can be accompanied by specific physicochemical properties and may result in an increased potential for crossing biological barriers, this may result in differences in behavior and (internal) exposure to the NMs (Bleeker et al., 2013; Lövestam et al., 2010).

Several NM based products are currently available and new NMs are constantly being developed and introduced in the market (Lövestam et al., 2010; Vance et al., 2015). The sources for NPs into the environment can be both natural and anthropogenic (Biswas and Wu, 2005; Buzea et al., 2007). The natural sources of NPs can be dust storms, forest fires,

volcanoes and ocean/water evaporation. The anthropogenic sources include diesel and engine exhaust, indoor pollution, cigarette smoke, buildings demolition, cosmetics and other consumer products and engineered NMs (Buzea et al., 2007).

Once in the environment, NPs can change during exposure, e.g. agglomerates or aggregates may form or disintegrate, particles may bind to other kinds of materials, coatings may be formed or disintegrate, particle surface area may change due to chemical reactions like oxidation increasing the difficulty in predicting their behavior and toxicity (Bleeker et al., 2013). This changes are related with the inherent properties of the NMs, like the solubility in water, colloidal stability and reactivity (Garner and Keller, 2014). Also the environmental matrix (air, soil, freshwater) where the NPs are can influence their toxicity. The chemical and physical characteristics of the environment surrounding the NPs can have a significant impact on the material behavior, which is especially important when evaluation the potential effects in biological systems (Lövestam et al., 2010). Properties of the matrix, such as ionic strength (IS), pH, organic matter (OM), and compartment composition can modify NMs behavior like aggregation and dissolution (Lowry et al., 2012; Tourinho et al., 2015). NPs are found in the different compartments (soil, air, water and landfills) (Bour et al., 2015). In particular, soils are a mixture of gas, liquid, and soil phases, interfaces between them, organic matter and microbial communities which make very difficult to understand the processes affecting the fate of NMs in this complex matrix (Tourinho et al., 2015). Tourinho et al (2015) refers that NMs in soil are likely to aggregate, sorb to surfaces, sediment, or/and dissolve. Transport of NPs in porous media, such as soil can happen and is related with sedimentation due to gravity, direct interaction of NPs with the soil or diffusion due to Brownian motion (Garner and Keller, 2014).

The limited information about NPs production and emissions into the environment limits broad conclusions about NPs in soils and effects in soil organisms, hence identification of patterns of fate and transformation processes of NPs in the environment.

1.4.3. State of the art: ecotoxicology of NiNPs

The broad applications of NiNPs increase the potential risk of human and environmental exposure and thus the risks related to their toxicity. The contributing factors to NPs toxicity are their size, chemical composition, shape, particle aging, and surface charge (Ahamed and Alhadlaq, 2014). Being smaller than cellular organelles and cells NiNPs can potentially penetrate basic biological structures, which may in turn disrupt their normal function (Magaye and Zhao, 2012). The toxicity of NPs is related with their persistence within the organisms through different exposure pathways or removal from the organisms due to immune system response leading to different dose-responses (Garner and Keller, 2014).

So far, most of the studies with NiNPs are focused on cell lines providing indications that NiNPs can induce genotoxicity, oxidative stress and cytotoxicity (Ahamed and Alhadlaq, 2014; Ahamed, 2011; Khan et al., 2013; Magaye and Zhao, 2012; Phillips et al., 2010). Among the few published ecotoxicological studies, nearly all of them are performed with organisms from the aquatic compartment such as *Danio rerio*, *Daphnia pulex*, *Ceriodaphnia dubia* and the algae *Pseudokirchneriela subcapitata* (Griffitt et al., 2008; Ispas et al., 2009b; Jayaseelan et al., 2014) evaluating parameters such as mortality, developmental defects, oxidative stress and growth inhibition.

Jayaseelan et al (2014) evaluated the oxidative stress and antioxidant response in the liver, gill and skin of the Mozambique tilapia *Oreochromis mossambicus* after exposure to NiNPs (56 nm spheres), showing an increase in SOD and POD activity and a reduction in CAT activity. They also showed that gills were the most sensitive organ presenting degeneration in muscle bundles, splitting of the muscle fiber, cellular damage and necrosis. A study with the sea urchin *Paracentrotus lividus* showed that 3 mg NiNPs/L (48 nm) significantly reduced embryos growth (Kanold et al., 2016). Ispas et al., (2009a) compared the effects of different sizes NiNPs in *D. rerio* and showed that there were differences in toxicity between them. The 30, 60, 100 nm and dendritic particles of aggregated 60 nm NiNPs caused 50 % lethality in fish embryos at 328, 361, 221 and 115 mg/L respectively, being the dendritic structures the most toxic from. A study with adult zebrafish (*D. rerio*) showed that 10 mg NiNPs/L did not cause acute toxicity to the fish (in 48 h), but NiNPs

were acutely toxic to aquatic invertebrates with LC_{50} of 3.89 mg NiNPs/L for *Daphnia pulex* (48 h test) and 0.35 mg NiNPs/L for *P. subcapitata* (96 h test) (Griffitt et al., 2008). The knowledge on the NiNPs effects on soil living invertebrates is limited to one study with *Eisenia fetida* (Heckmann et al., 2011) which reported a 16% reduction in juvenile production at 1000 mg NiNPs/kg.

1.5 Main goals and outline of the thesis

The main aim of this work is to compare the effects of Nickel nanoparticles in comparison with Nickel salt ($NiNO_3$) on *Enchytraeus crypticus*, based on the standard ERT and in the FLC test.
The present thesis is organized in three chapters.

Chapter 1: General introduction

Chapter 2: Hazard assessment of NiNPs in soil – the use of a full life cycle test with *Enchytraeus crypticus* – no monotone dose response? (Fátima C.F. Santos, Susana I.L. Gomes, Janeck J. Scott-Fordsmand and Mónica J.B. Amorim)

Chapter 3: General discussion and final considerations

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Chapter 2

Hazard assessment of NiNPs in soil – the use of a full life cycle test with *Enchytraeus crypticus* – no monotone dose response?

Hazard assessment of NiNPs in soil – the use of a full life cycle test with *Enchytraeus crypticus* – no monotone dose response?

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Abstract

Nanoparticles (NPs) such as nickel (Ni) are currently widely used in several applications, with an estimated production of 20 tons/year just in the US. Nevertheless the environmental effects of NiNPs are still poorly understood. In the present study, the toxicity of NiNPs and $NiNO_3$ was assessed using the soil standard model *Enchytraeus crypticus* (Oligochaeta) in a Full Life Cycle (FLC) test, adding the endpoints hatching, growth and time to reach maturity, besides survival and reproduction as in the standard. Overall, $NiNO_3$ was more toxic than NiNPs and toxicity seems to occur via differentiated mechanisms. Exposure to $NiNO_3$ showed effects that started on reduced hatching and then remained throughout the life cycle in all the measured endpoints (growth, maturity reaching, survival and reproduction). For NiNPs, hatching was the most sensitive endpoint (EC_{10}=47 mg Ni/kg), although, the additional endpoints across the life cycle show that this corresponded to a delay in hatching and organisms survived and reproduced at concentrations up to 1800 mg NiNPs/kg. Interestingly, the lowest tested concentration of NiNPs (100 mg NiNPs/kg) caused effects similar to the higher (1000 and 1800 mg Ni/kg), indicating higher nano-particulate effect. The following highlights the potential lack of monotone dose-response, at least based on mass, for hazard assessment of NPs and hence the requirement of a revised procedure for Risk Assessment.

Keywords: nanomaterials; long-term; oligochaeta; life stages

1. Introduction

Nickel (Ni) is a naturally occurring element in the earth's crust, being present in soils in concentrations from 3 up to 1000 mg Ni/kg soil (Iyaka, 2011) and can reach 26000 mg/kg in heavily polluted areas (Alloway, 1995). It is widely used in industry (e.g. mechanics, electronic (Denkhaus and Salnikow, 2002), catalysis, jewelry (Iyaka, 2011)). Nickel as nanoparticles (NiNPs) have been introduced in various consumer products and industries, e.g. catalysis, ceramics, lubricants, coatings, electrical, fuel, (NiPERA, 2013), with an estimated production around 20 tons per year just in the United States (NiPERA, 2013). Studies on the effects of Ni salt ($NiCl_2$) showed effects on survival and reproduction of different soil invertebrate species including the earthworms *Eisenia fetida* (Lock and Janssen, 2002) and *Eisenia veneta* (Scott-Fordsmand et al., 1998); the collembolans *Folsomia candida* (Lock and Janssen, 2002) and *Folsomia fimetaria* (Scott-Fordsmand et al., 1999) and the enchytraeid *Enchytraeus albidus* (Gomes et al., 2014; Lock and Janssen, 2002) reporting 50% reproduction effect concentrations (EC_{50}) ranging between 60 mg Ni/kg for *E. albidus* (Gomes et al., 2014) to 450 mg Ni/kg for *F. fimetaria* (Scott-Fordsmand et al., 1999) and *F. candida* (Lock and Janssen, 2002). For NiNPs very little is known for soil living invertebrates with only one study using *E. fetida* (Heckmann et al., 2011) reporting a 16% reduction in juvenile production at 1000 mg Ni/kg.

Most of the studies used cell lines showing genotoxicity and carcinogenity (Magaye and Zhao, 2012; Magaye et al., 2014), oxidative stress (Ahamed and Alhadlaq, 2014; Ahamed, 2011; Jayaseelan et al., 2014) and cytotoxicity (Alarifi et al., 2014; Phillips et al., 2010). From an ecotoxicological point of view, the majority of the information available focus on aquatic organisms. For instance, Ispas et al. (2009b) showed that NiNPs of several sizes and shapes caused 50% mortality in zebra fish (*Danio rerio*) between 115 and 360 mg Ni/L which is in the same range as Ni-salt, while *Daphnia pulex* and the algae *Pseudokirchneriela subcapitata* were more sensitive (LC_{50} of 3.89 and 0.35 mg Ni/L, respectively) (Griffitt et al., 2008). For overview, information is summarized in Table 1.

Table 1: Summary of literature review of toxicity studies for nickel nanoparticles, indicating the biological entity, size and morphology of NiNPs, exposure media, measured endpoint, EC_{50} or other effects and the respective references.

Biological entity	**NiNPs** (size (nm), morphology)	**Exposure media**	**Endpoint**	**EC_{50} (mg/l) / Other**	**Reference**
Cell lines: Human lung epithelial A549 cells	70	DMEM/F-12 medium	-Cytotoxicity -Oxidative stress	↑Cell death ↑Cell membrane damage ↑ROS ↑LPO ↓Glutathione ↑Apoptosis	(Ahamed, 2011)
Cell lines: Human breast carcinoma MCF-7 cells	28	DMEM medium	-Cytotoxicity -Genotoxicity	↓Cell viability ↑Cell membrane damage ↑ROS ↓Glutathione ↑Apoptosis ↑DNA damage	(Ahamed and Alhadlaq, 2014)
Cell lines: Mouse epidermal JB6 cells	80	5% FBS EMEM medium	-Carcinogenity	Activation of tumor promotion factors (AP-1 and NF-KB) ↓p53 activation	(Magaye et al., 2014)
Cell lines: Human skin epithelial cells A431	52	DMEM/F-12 medium	-Cytotoxicity	↑Cell membrane damage ↑ ROS ↓ Glutathione ↑LPO ↑SOD activity ↑CAT activity ↑ Apoptosis ↑ DNA Damage	(Alarifi et al., 2014)
Oreochromis mossambicus	56, spheres	Water (freshwater)	-Oxidative stress -Antioxidant response -Histopatho-logical changes in gills	↑SOD activity ↑POD activity ↓CAT activity Degeneration in muscle bundles Necrosis Splitting of muscle fiber	(Jayaseelan et al., 2014)
Danio rerio	30, spheres	water (freshwater)	Survival	328	(Ispas et al., 2009)
	60, spheres			361	
	100, spheres			221	
	60, dendritic			115	
Danio rerio	5-20	Water (freshwater)	Survival	>10	(Griffitt et al., 2008)
Daphnia pulex		Water (freshwater)	Survival	3.98	
C. dubia		water (freshwater)	Survival	0.674	
P. subcapitata		Water (freshwater)	Growth inhibition	0.35	
Paracentrotus lividus	48	water (seawater)	Embryonic development	3	(Kanold et al., 2016)
Eisenia fetida	40.4 ± 1.13	soil	Reproduction	16% reduction	(Heckmann et al., 2011)

Concerns have been highlighted regarding the need for longer-term effects of NMs (e.g. Kumar et al., 2014), often short and not covered in the currently available standard toxicity tests. The recently a full life cycle (FLC) test developed for the soil model organism *Enchytraeus crypticus* (Oligochaeta, Enchytraeidae) (Bicho et al., 2015) represents a longer exposure test and adds further endpoints to the standard Enchytraeid Reproduction Test (ERT) survival and reproduction (ISO, 2004; OECD, 2004): hatching, growth, and time to reach maturity. Organisms are exposed from cocoon stage (1-2 days old) until next generation juveniles (46 days), hence effects are traced on potentially more sensitive life stages (e.g. embryonic development) and, also the time coverage is much wider compared to the standard toxicity test (21 days).

The aim of the present study was to assess the toxicity of NiNPs and $NiNO_3$ using the FLC test with *E. crypticus* and compare the results with the standard ERT.

2. Materials and Methods

2.1. Test organism

Enchytraeus crypticus (Enchytraeidae, Oligochaeta), Westheide & Graefe, 1992 was used. The cultures were kept in agar, consisting of Bacti-Agar medium (Oxoid, Agar No. 1) and a sterilized mixture of four different salt solutions ($CaCl_2 \cdot 2H_2O$; $MgSO_4$; KCl; $NaHCO_3$) at the temperature of 19±1ºC with photoperiod of 16:8 hours light: dark. Cultures were fed on grinded autoclaved oats twice per week.

2.2. Test materials and characterization

Nickel nanoparticles (NiNPs) (American Elements) and Nickel nitrate ($Ni(NO_3)_2.6H_2O$, Fluka, ≥ 98.5%) (Table 2) were used. Further details can be found in (Heckmann et al., 2011).

Table 2: Characteristics of the tested NiNPs materials and $NiNO_3$ including manufacturer, CAS, size, nominal surface area, purity, crystallinity and solubility/dispersability.

	NiNPs	**$NiNO_3$**
Manufacturer	American Elements	Fluka
CAS number	7440-02-0	13478-00-7
Size (nm) (nominal)	20	-
Crystallite (nm)	38.8	-
TEM (nm)	40.4 ± 1.13	-
DLS	3867 ± 211	-
Nominal surface area (m^2/g)	30–50	-
Purity (%)	99.9	98.5
Crystallinity (PXRD)	Cubic	-
Solubility/Dispersability	Not dispersible in water	Water soluble

2.3. Test soil and spiking procedures

The natural standard LUFA 2.2 soil (Speyer, Germany) was used, having the following main characteristics: pH (0.01 M $CaCl_2$) = 5.5; organic carbon = 1.61 %, cation exchange capacity (CEC) = 10.0 meq/100g, maximum water holding capacity (WHC) = 43.3 %, and grain size distribution of 7.9% clay (< 0.002 mm), 16.3 % silt (0.002 - 0.05 mm) and 75.8 % sand (0.05 – 2.0 mm).

Spiking of the soil was performed to reach the following concentration range: 0-10-32-100-320-1000 mg Ni/kg for $NiNO_3$ and 0-100-400-700-1000-1500 mg Ni/kg for NiNPs for the ERT. For the FLC teste concentration range was: 0-3.2-10-32-100-320 mg Ni/kg for $NiNO_3$ and 0-100-320-1000-1800-3200 mg Ni/kg for NiNPs. The OECD guidelines (OECD, 2004) for the testing of non-soluble substances were followed: NiNPs were added as dry powder to the dry soil and mixed manually, after which water was added until 50% of the soil WHC. Spiking was done per individual replicate (except for the lowest concentration where the 4 replicates were mixed together due to increased error in weighing).

For $NiNO_3$ the OECD guideline (OECD, 2004) for the testing of soluble substances was followed: $NiNO_3$ was added to pre-moistened soil as aqueous solutions .

2.4 Test procedures

2.4.1. Enchytraeid reproduction test – ERT

The test procedures followed the ERT (OECD, 2004). In short, 10 adult enchytraeids with well-developed clitellum and similar size were introduced in each test vessel containing 20 g of moist soil and 25 mg of food (autoclaved grinded oats). Test ran for 3 weeks at 20±1°C and photoperiod of 16:8 h (light: dark). During the test duration, food (12 mg) and water content (based on weight loss) were replenished weekly. Four replicates per treatment were used. At the test end, for counting of organisms, the organisms were fixated with ethanol and coloured with Bengal rose (1% in ethanol). After 24 h, soil samples were sieved through meshes with decreasing pore size (1.6, 0.5 and 0.3 mm) to separate the enchytraeids from most of the soil and facilitate counting. Adult and juvenile organisms were counted using a stereo microscope and survival and reproduction assessed.

2.4.2. Full life cycle test - FLC

The full life cycle (FLC) test was performed according to the procedures described in Bicho et al. (Bicho et al., 2015). Endpoints assessed included hatching success (day 11), growth (day 11, 15, 22, 25), maturity status (day 25), survival and reproduction (day 46). In short, the test starts with cocoons (1-2 days old) selected from synchronized cultures. Ten cocoons were introduced in each test vessel containing 10g of moist soil and the test ran at 20±1°C with 16:8 h (light:dark) photoperiod. Four replicates per treatment were used. Food (6 mg autoclaved grinded oats) was added for the first time at day 11 and then replenished weekly together with water content (based on weight loss). At each sampling point, organisms were counted following the described method. A sub-sample of the organisms in each replicate (n=20) was measured for length.

2.5. Data analysis

To assess differences between control and Ni treatments, One-Way Analysis of Variance (ANOVA) was performed followed by Dunnett´s method for multiple comparisons at a significance level of 0.05 (SigmaPlot 11.0). The effect concentrations (EC_x) were estimated using the Toxicity Relationship Analysis Program (TRAP), excluding the 100 mg/kg result for the NiNPs model fit.

3. Results

3.1 Enchytraeid Reproduction Test (ERT)

The validity criteria was fulfilled as within the standard guideline (OECD, 2004): in controls adults' mortality was lower than 20%, the number of juveniles higher than 25 and the coefficient of variation lower than 50%. There were no significant changes in soil pH within the test conditions or over the test duration (pH=5.19±0.1 and 5.20±0.1 for $NiNO_3$ and 5.22±0.02 and 5.12±0.03 for NiNPs at the beginning and end of the test, respectively). Results showed a higher toxicity of $NiNO_3$ compared to NiNPs (Fig. 1). The ECx values are presented on Table 3.

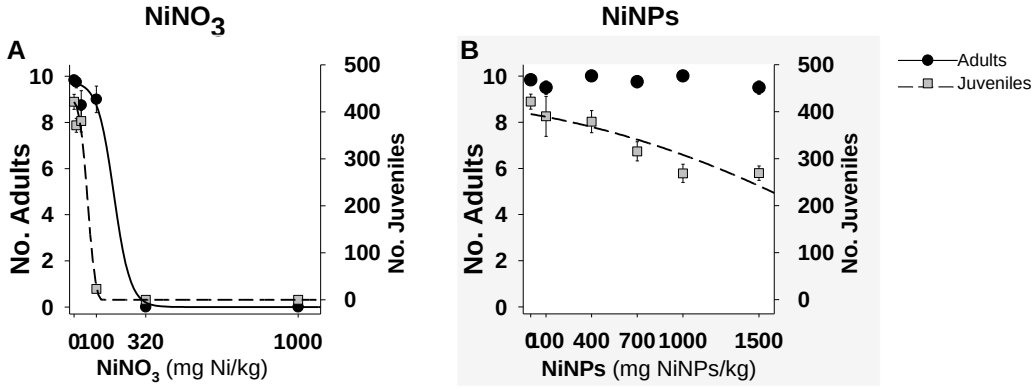

Figure 1: Results in terms of survival and reproduction (Enchytraeid Reproduction Test) for *Enchytraeus crypticus* exposed to nickel nitrate ($NiNO_3$) and nickel nanoparticles (NiNPs). Results are expressed as average ± standard error. The solid and dashed lines represent the model fit to data**.**

3.2. FLC test

The soil pH within test conditions was 5.35±0.1 and 5.69±0.2 for $NiNO_3$ and 5.3±0.03 and 5.8±0.2 for NiNPs at the beginning and the end of the test, respectively, which represent normal pH variations. The results can be depicted in Figure 2 and the EC values on Table 3.

3.2.1. Hatching

There was a dose-dependent decrease in the number of hatched juveniles for both materials (Fig. 2A,B), although for $NiNO_3$ hatching was fully inhibited at ≥100 mg Ni/kg and for NiNPs for 100 mg NiNPs /kg the effect was also exceptionally higher.

3.2.2. Growth

$NiNO_3$ exposure reduced growth in all sampling times. There are no measurements for the concentrations 100 and 320 mg Ni/kg (except day 11) due to absence of organisms. For NiNPs exposure organisms' growth was affected at days 15 and 22.

3.2.3. Maturity Status

$NiNO_3$ caused a decrease in the percentage of clitellate adult organisms. Exposure to NiNPs caused no significant effects in the maturity status.

3.2.4. Survival and reproduction

$NiNO_3$ caused a dose-dependent decrease in the number of surviving adults and juveniles produced. NiNPs significantly reduced adults' survival at the concentrations 100 and 3200 mg Ni/kg and reproduction was negatively affected in the highest concentration (3200 mg Ni/kg).

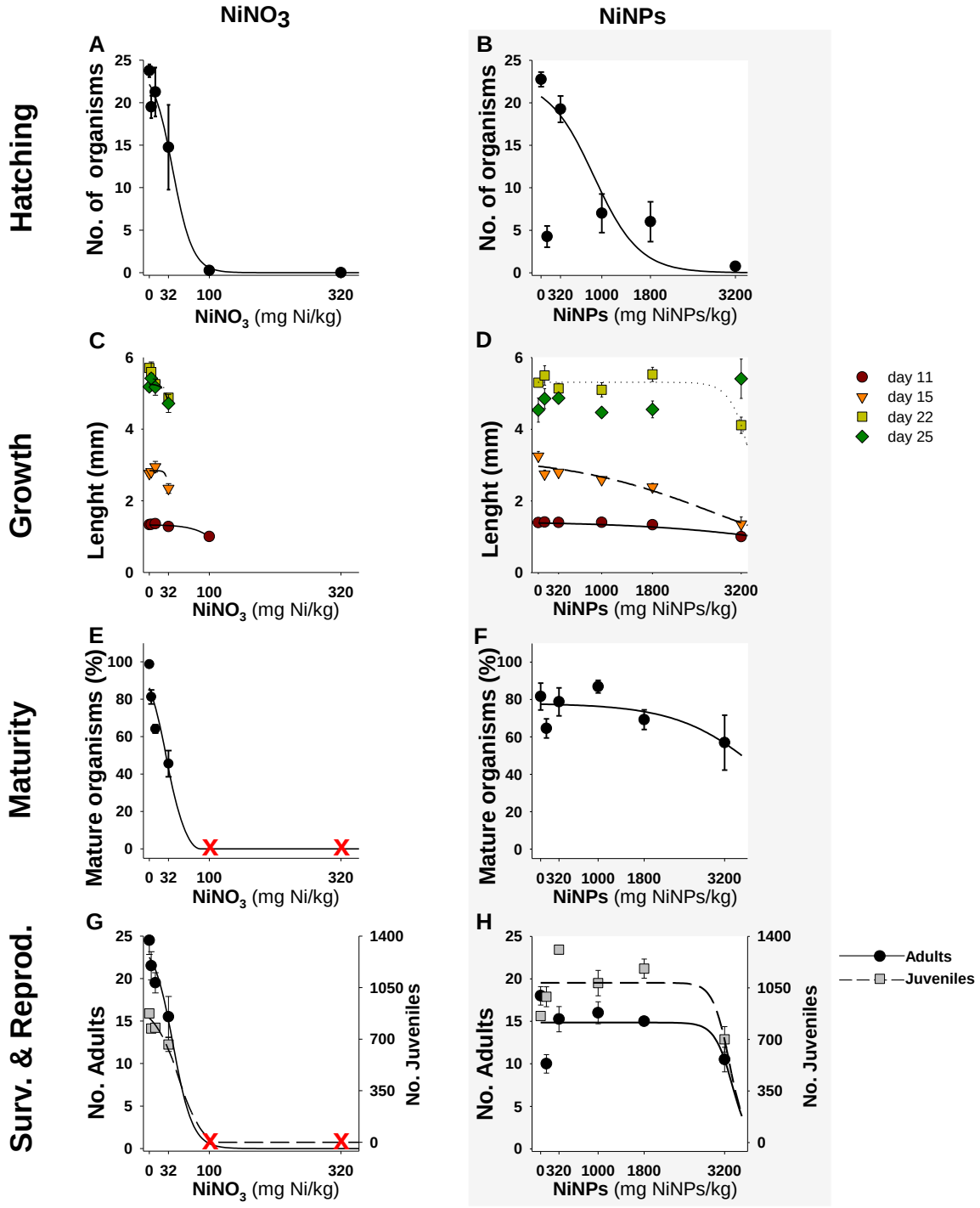

Figure 2: Results in terms of hatching (A,B), growth (C,D), maturity status (E,F), survival and reproduction (G,H) for *Enchytraeus crypticus* exposed to nickel nitrate ($NiNO_3$) and nickel nanoparticles (NiNPs) during the Full Life Cycle (FLC) test. Results are expressed as average ± standard error. The solid and dashed lines represent the model fit to data. X: no organisms.

Table 3: Summary of the effect concentrations (ECx) for *Enchytraeus crypticus* exposed to nickel nanoparticles (NiNPs) and nickel nitrate ($NiNO_3$). ECx and the 95% confidence intervals are shown with the respective models used to fit data including the values for slope (S) and top point (Y0). EC_x values are given per endpoint and test type. ERT: Enchytraeid Reproduction test; FLC: Full life cycle test; d: days; CI: confidence intervals; n.e.: no effect; n.d.: not determined; Log 2 param: Logistic 2 parameters; Log 3 param: Logistic 3 parameters; Thres sig 2 param: Threshold sigmoid 2 parameters.

			$NiNO_3$					**NiNPs**				
Test	**Endpoint**	**Time (days)**	**EC_{10}**	**EC_{50}**	**EC_{90}**	**Model**	**(Parameters)**	**EC_{10}**	**EC_{50}**	**EC_{90}**	**Model**	**(Parameters)**
ERT	Surv	21d	98 (66-130)	178 (125-231)	258 (163-354)	Log 2 param	(S:4.8E^{-03}; Y0:9.8)	n.e	n.e	n.e	-	-
	Repr	21d	26 (17-36)	61 (53-69)	82 (61-104)	Thres sig 2 param	(S:1.4E$^{-02;}$ Y0:421)	243 (-126-612)	1704 (1313-2096)	2607 (1867-3347)	Thres sig 2 param	(S:3.5E^{-04}; Y0:421)
FLC	Hatching	11d	2.6 (-83-88)	39 (17-61)	75 (4-147)	Log 3 param	(S:1.51E^{-02}; Y0:24.2)	47 (-410-503)	870 (643-1096)	1692 (1175-2210)	Log 2 param	(S:6.68E^{-04}; Y0:22.75)
	Growth	11d	76 (57-93)	124 (102-147)	173 (114-231)	Log2 param	(S:1.13E^{-02}; Y0:1.33)	1672 (725-2619)	4565 (2652-6478)	7458 n.d	Log 2 param	(S:1.90E^{-04}; Y0:1.43)
		15d	30 (-94-153)	37 (-257-331)	44 (-668-756)	Log 2 param	(S:7.97E^{-02}; Y0:2.8)	183 (-334-700)	2838 (2838-2496)	5494 (4601-6387)	Log 2 param	(S:2.07E^{-04}; Y0:3.2)
		22d	26 (18-34)	60 (31-88)	92 (35-150)	Log 2 param	(S:1.66E^{-02}; Y0:5.65)	3030 (-13445-19505)	3415 (-17450-24280)	3801 n.d.	Log 2 param	(S:1.43E^{-03}; Y0:5.3)
		25d	32 (26-37)	47 (-18-112)	62 (-67-192)	Log 2 param	(S:3.62E^{-02}; Y0:5.26)	n.e	n.e	n.e	-	-
	Maturity	25d	<3.2	28 (23-33)	48 (35-60)	Thres sig 2 param	(S:1.76E$^{-02;}$ Y0:98.8)	2270 (586-3953)	3946 (2158-5734)	5622 (880-10365)	Log 2 param	(S:3.28E^{-04}; Y0:77.9)
	Surv	46d	4 (-8-16)	40 (29-50)	75 (49-102)	Log 2 param	(S:1.53E^{-02}; Y0:24.5)	3121 (-43544-49787)	3609 n.d.	3551 n.d.	Log 3 param	(S:5.4E^{-04}; Y0:14.85)
	Repr	46d	12 (5-18)	49 (42-56)	72 (52-92)	Thres sig 2 param	(S:1.48E$^{-02;}$ Y0:875)	3088 (-79583-85761)	3455 n.d.	3481 n.d.	Log 3 param	(S:5.88E^{-04}; Y0:1084)

4. Discussion

Based on the standard ERT, $NiNO_3$ toxicity was within the values previously reported for *E. albidus* (Gomes et al., 2014). NiNPs were 10 to 30 times less toxic in terms of reproduction EC_{10} and $EC_{50/90}$, respectively. Several studies on metallic NPs have reported higher toxicity of the salt form in comparison to the respective metallic NPs, e.g. in soil oligochaetes, this was the case for Cu and Ag to *E. fetida*, (Heckmann et al., 2011), Ag to *E. albidus* (Gomes et al., 2013) and Cu to *E. crypticus* (Gomes et al., 2015). Heckmann et al. (2011) reported 16% reproduction inhibition for *E. fetida* at 1000 mg NiNPs/kg, hence *E. crypticus* seems to be more sensitive (EC_{10} = 243 (-126 – 612) mg/kg). Based on the *E. crypticus* FLC test results, $NiNO_3$ was also more toxic than NiNPs. Hatching was the most sensitive endpoint, although this was a delay in hatching and not a "no hatch" result (at day 25 the number of organisms was similar to control, except for 3200 mg NiNPs/kg). Ispas et al. (Ispas et al., 2009b) reported increased embryo mortality and malformations (in *D. rerio*) after exposure to NiNPs of several sizes and shapes (spheres and dendrites), but the exposure was done in dechorionated embryos, which increased the exposure of the embryos to the NPs and possibly influenced the way NPs were uptaken.

The lowest tested concentration (100 mg NiNP/kg) caused similar effects to the highest, e.g. 1000 and 1800 mg Ni/kg. This could be related with less aggregated NPs in the lower concentrations hence higher toxicity, as described for other metallic NPs in aquatic media, e.g. copper (Griffitt et al., 2007), silver (Yang et al., 2012) and NiNPs (Ispas et al., 2009b; Jayaseelan et al., 2014). In soil, a study by Klitze et al. (Klitzke et al., 2015) showed that, at higher concentrations AgNPs formed larger aggregates in soil solution than at lower concentrations. Release of Ni ions from NiNPs was reported by Pietruska et al. (Pietruska et al., 2011) in cell cultures media. In our study, at the lowest concentration (100 mg Ni/kg) NiNPs would have been less aggregated and hence more reactive to cause adverse effects on *E. crypticus*. Additionally, the release of Ni ions could have contributed to the higher toxicity ($NiNO_3$ was very toxic to *E. crypticus* embryos (hatching EC50 = 39 mg Ni/kg).

Inhibition of growth caused by NPs exposure was reported for several organisms, e.g. in *Caenorhabditis elegans* exposed to AgNPs (Meyer et al., 2010), TiO_2 and ZnO-NPs (Wu

et al., 2013); in *D. rerio* and *Daphnia magna* exposed to TiO_2-NPs (Chen et al., 2011; Zhu et al., 2010).

Our results indicate that *E. crypticus* growth was delayed for NiNPs exposure, and at day 22 there were no differences in organisms' length. $NiNO_3$ caused more severe and permanent effects on growth, since organisms were not able to recover throughout the lifecycle (organisms remained smaller until reaching maturity). On the contrary to NiNPs, juvenile stage was very sensitive to $NiNO_3$. This is in line with the literature data reporting growth inhibition in response to $NiNO_3$ exposure (e.g. in *Folsomia fimetaria* (Scott-Fordsmand et al., 1999). NiNPs reduced the maturation of *E. crypticus* (in the highest concentration tested). Since we know the growth development of juveniles was not affected by NiNPs, the reduction in maturity status must be caused by specific effects on reproductive tissues/organs. Kong et al. (2014) described inflammation and apoptosis in ovary tissues and changes in sperm motility in (female and male) mice exposed to NiNPs, linked to reduction in reproduction and in the size of the offspring. Our results showed that the reduction on reproduction (at day 46) could be consequential of the reduction in the maturation observed at day 25. This points to an impairment in reproduction capacity due to effects on reproductive system. For $NiNO_3$, the maturity status was among the most sensitive endpoints (lowest EC_{50} = 28 mg Ni/kg), and in line with the developmental growth delay observed in the juvenile stage, with the organisms not able to reach maturity at day 25. That delay can partly explain the reduction in reproduction observed at day 46. Comparing the ERT and FLC test results, the reproduction ECx values were surprisingly lower in the ERT for NiNPs. We suggest 2 hypothesis to support this results: 1) in the FLC test, the exposure from cocoon stage could act as some kind of selection factor for the most resistant, hence lower toxicity values in the posterior life-stages and/or 2) the effect of NiNPs occurs at the pre-reproductive stage or the step where cocoons are layed, hence the ERT results show higher effects, i.e. the adult worms are exposed from the start and to the result can be laying less cocoons or lay cocoons with malformations, not viable etc. For $NiNO_3$, the ECx values for the different endpoints of the FLC test were very similar. In the literature we can find information about the effects of $NiNO_3$ in different endpoints (e.g. embryo lethality in *Gastrophryne carolinensis* (Fort et al., 2006); growth decrease in *Folsomia fimetaria* (Scott-Fordsmand et al., 1999); negative effects on survival and reproduction for several soil invertebrates (Gomes et al., 2014; Lock and Janssen, 2002;

Scott-Fordsmand et al., 1999, 1998), but never assessed throughout the life cycle of an organism and thus not covering the different endpoints for the same species. The FLC test results indicate that the effects caused by $NiNO_3$ at early life stages (embryos inside the cocoons) persist over *E. crypticus* development causing growth impairment, decrease in the proportion of mature adults and ultimately higher mortality rates in adults organisms exposed since the cocoon stage. Given the EC_{50} values for survival were 4.5 times higher for the ERT than for the FLC test (178 and 40 mg Ni/kg, respectively), long-term exposure to $NiNO_3$ poses higher risks to enchytraeids' populations than predicted based on the standard ERT.

5. Conclusions

$NiNO_3$ was more toxic than NiNPs and toxicity seems to occur via differentiated mechanisms. Exposure to $NiNO_3$ showed effects that started on reduced hatching and then remained throughout the life cycle in all the measured endpoints. For NiNPs, hatching was the most sensitive endpoint, but this corresponded to a delay in hatching and organisms survived and reproduced at concentrations up to 1800 mg NiNPs/kg. The lowest tested NiNPs concentration (100mg/kg) caused a much higher effect than predicted based on a mass basis (concentration-response), probably due to increased nano-particulate effect compared to the higher concentrations where aggregation increased. The following highlights the potential lack of monotone dose-response, at least based on mass, for hazard assessment of NPs and hence the requirement of a revised procedure for Risk Assessment.

Acknowledgments

Thanks are due, for the financial support to the European Commission within FP7-SUN: SUstainable Nanotechnologies (Ref. 604305), CESAM (UID/AMB/50017), to FCT/MEC through national funds, the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020, and a post-doc grant to Susana Gomes (SFRH/BPD/95775/2013).

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Chapter 3

General discussion and final considerations

General discussion and final considerations

Several NM base products are being developed and applied in a wide range of products and it is likely that they will end in the several compartments of the environment (including soils), where they can cause harmful effects. Because of the complexity of soil matrix and its characteristics (pH, OM, IS), the NMs properties (e.g. size, chemical composition, surface charge, dissolution rate, aging), the uptake and elimination rates in the organisms and the lack of global knowledge about production, applications and emissions, it can be very difficult to predict the behavior of NPs and their effects to the environment and the living organisms.

Despite soil compartment being a final destination of NMs there is little information regarding the effects of NMs in this matrix and to their inhabitants. This is especially concerning regarding longer-term studies.

In this thesis, the effects of NiNPs and $NiNO_3$ to the soil model *E. crypticus* were investigated using a Full Life Cycle test (longer and more comprehensive in comparison to the standard Enchytraeid Reproduction Test), filling a current gap regarding the assessment of effects of NPs in soil compartment.

Both $NiNO_3$ and NiNPs cause adverse effects throughout *E. crypticus* life cycle, being $NiNO_3$ more toxic than NiNPs. The toxicity seems to occur via different mechanisms. For $NiNO_3$, the effects observed on hatching, remained throughout all endpoints evaluated in the life cycle, where dose-dependent effects where observed. For NiNPs, there was a delay in hatching, being this the most sensitive endpoint. Nevertheless organisms where able recover and survive and reproduce at concentrations up to 1800 mg NiNPs/kg. The effects observed at 100 mg NiNPs/kg where higher than predicted on a mass basis, which could be explained by an increase in nano-particulate effect compared to higher aggregation that occur in higher concentrations. Aggregated NPs interact with the organisms in a different way and at different rates when compared to single NPs and dissolved ions. In the present study, higher NP concentrations should have increased the formation of NPs aggregates hence less available to cause negative effects to the organisms. This resulted in the absence of a monotone dose-response, which highlights the need to a revised procedure for Risk Assessment of NPs/NMs.

This work highlighted the importance of testing NMs (in soil organisms) using a more comprehensive test (FLC) which allow a more in-depth study on different organisms' life stages and testing duration. This way, providing a better understanding of possible mechanisms of NPs toxicity over time.

Nevertheless, a lot of work developing strategies, tools, and policies are still needed to ensure a safe and responsible production/use of NMs.